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GRANT NUMBER DAMD17-94-J-4296

TITLE: The Role of Oxidative Stress in Apoptosis of Breast Cancer Cells

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REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (29 Aug 96 - 28 Aug 97)

4. TITLE AND SUBTITLE
The Role of Oxidative Stress in Apoptosis of Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-94-J-4296

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

In response to specific stimuli, normal cells undergo a process of cell suicide called apoptosis. This project is testing the hypothesis that oxidative stress plays a critical role in the mechanism of apoptosis induced by treatment of human breast cancer cells with tumor necrosis factor-α (TNF). TNF treatment of MCF-7 cells was found to increase the expression and activity of manganese superoxide dismutase. Expression of other antioxidant defense genes and catalase activity did not change with TNF treatment. We could not detect oxidative damage to lipids in TNF-treated cells, using an assay for malondialdehyde. Transfectants with increased antioxidant activity are being analyzed to test our hypothesis. Reliable measurements of apoptosis are critical for this analysis and several different methods were compared. Methods based on detection of DNA fragmentation in intact cells did not work well in our system. Counting apoptotic cells based on morphology was found to be reliable, but the amount of apoptosis seen at any time was small. Cells stably transfected with DT-diaphorase were not consistently different in their TNF sensitivity. One transfectant appeared more sensitive to TNF; this clone also showed decreased superoxide dismutase and catalase activity. Analysis of catalase transfectants is ongoing.

14. SUBJECT TERMS
Breast Cancer, apoptosis, oxidative stress, MCF-7 cells, tumor necrosis factor-alpha, antioxidant defenses, transfectants

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Margaret Burke 9/26/97

PI - Signature Date

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INTRODUCTION

In response to specific stimuli, normal cells undergo a process of cell suicide which has been termed programmed cell death, or apoptosis (1). Signals that trigger specific cells to undergo apoptosis include insufficient growth factors, cytokines, steroid hormones and exposure to DNA-damaging agents. The control of cell populations by apoptosis is important for homeostasis and for the removal of damaged, potentially harmful, cells. A change in the control of apoptosis can lead to diseases of excessive cell growth (e.g., cancer) or excessive cell loss (e.g., autoimmune disorders) (2,3). Understanding the molecular mechanism of apoptosis is expected to lead to new strategies for treating these diseases. We are particularly interested in applying an understanding of apoptosis to improving the treatment of breast cancer.

Preliminary studies led me to the hypothesis that the control of oxidative stress plays a critical role in the mechanism of apoptosis (reviewed in ref. 4). Oxidative stress reflects an imbalance in the cell between the rates of production and removal of reactive oxygen species (5,6). Reactive oxygen species are produced when electrons leak from the mitochondrial respiratory chain and other electron transfer systems and are captured by oxygen. Single electron reduction of oxygen successively generates superoxide anion radicals, hydrogen peroxide and hydroxyl radicals. These species can react with and cause damage to DNA, lipids and proteins.

Apoptosis of breast cancer cells is induced by tumor necrosis factor-α (TNF) (7). TNF is a 17.5 kD protein produced by activated macrophages and monocytes. It was isolated following the observation of tumor regression in cancer patients who contracted bacterial infections (8,9). While the mechanism by which TNF causes apoptosis is not yet entirely clear, oxidative stress appears to be involved. In some cell types, TNF cytotoxicity has been shown to require oxygen and is inhibited by free radical scavengers (10). The sensitivity of human embryonic kidney and cervical carcinoma cell lines to TNF has been modulated by transfection of sense and anti-sense sequences of the antioxidant defense gene, MnSOD (11,12).

Given that oxidative stress is critical to the mechanism of apoptosis, cellular defenses against oxidative stress will influence susceptibility to apoptosis. Copper,zinc-superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD), located in the cytoplasm and mitochondria, respectively, convert superoxide radical to hydrogen peroxide. Catalase and glutathione peroxidases convert hydrogen peroxide to water. DT-diaphorase (NAD(P)H:quinone (acceptor) oxidoreductase) may also protect against oxidative stress (13). One goal of this project is to determine whether altered expression of antioxidant defenses plays a role in the mechanism of TNF-induced apoptosis of breast cancer cells. Another goal is to test whether sensitivity of breast cancer cells to TNF can be modulated by using gene transfections to alter the strength of the antioxidant defense.

Studies with oxidative stress-responsive transcription factors are consistent with the idea that TNF treatment of cells results in oxidative stress. The activity of a number of transcription factors is sensitive to cellular redox state (14). NF-κB is activated under conditions of oxidative stress (15) and expression from AP-1 and NF-κB reporter constructs has been used as an *in vivo* indicator of oxidative stress (16,17). TNF treatment of the J. Jhan human lymphoblastoid T cell and U937 myelomonocytic cell lines increases expression from a NF-κB-CAT reporter construct (17). For this project, we proposed testing whether oxidative stress will activate the NF-κB and AP-1 transcription factors in breast cancer cells.

Hypothesis, specific aims and significance of this project

The **hypothesis** being tested here is *that oxidative stress plays a critical role in the mechanism of apoptosis induced by treatment of human breast cancer cells with TNF*. The **specific aims** for the project are to: 1) use Northern blot hybridization analyses and enzyme assays to define the extent of changes in the cellular antioxidant defense, after treatment of breast cancer cells with TNF; 2) determine whether breast cancer cells that have been transfected with expression vectors encoding antioxidant defense genes are resistant to TNF-induced apoptosis; 3) determine whether the induction of anti-sense antioxidant defense genes is sufficient to cause apoptosis of breast cancer cells, and 4) investigate the effects of oxidative stress, in breast cancer cells, on redox-sensitive transcription factors.

The proposed project is **significant** to the treatment of breast cancer because: 1) it is likely that defects in the apoptotic pathway contribute to the conversion of normal breast tissue to malignant cancers; 2) induction or restoration of apoptosis has promise as a novel approach to treating breast cancer; 3) an understanding of the underlying molecular mechanism is needed to guide the rational development of apoptosis-based breast cancer treatments, and 4) the proposed experiments test a specific hypothesis relevant to the mechanism of apoptosis in breast cancer cells.

BODY

EXPERIMENTAL METHODS

Cell culture. MCF-7 cells were maintained at 37°C under 5% CO_2 in either RPMI (Irvine Scientific, Santa Ana, CA) or low glucose Dulbecco's Modified Eagle's Medium. Cells used for biochemical analyses were grown in RPMI. Cells used for transfections were gradually adjusted to growth in DMEM, prior to the transfections, and were maintained from then on in DMEM. Except for RPMI, all cell culture reagents were obtained from Gibco BRL (Gaithersburg, MD). Media was supplemented with L-glutamine, 5% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Cultures were tested periodically and confirmed to be mycoplasma-free. For treatment with TNF, cells were plated and allowed to attach overnight (approximately 15 h). Recombinant human TNF (Boehringer Mannheim, Indianapolis, IN) was diluted in a small volume of media and then added to the cultures. Cells were collected from the media as well as the monolayer at the end of each treatment period.

Enzyme assays. The method described by Amstad et al. (18) was used to prepare cell lysates for SOD measurements. Briefly, the monolayer cells were washed three times with PBS, scraped from the plates and collected by centrifugation. The cell pellets were resuspended in 100 mM triethanolamine-diethanolamine, pH 7.4 and lysed, while on ice, using 3-10 sec. bursts from a VC40 Ultrasonic Liquid Processor (Sonics & Materials, Inc., Danbury, CT). The lysates were centrifuged at 100,000 x g and then dialyzed to remove small interfering substances. Samples were stored at -80°C until assayed. Total SOD activity was measured as described by Paoletti et al. (19). The method is based on the inhibition of a superoxide-driven NADH oxidation. The final assay mixture contained 88 mM triethanolamine-diethanolamine, pH 7.4, 2.4 mM EDTA, 1.2 mM $MnCl_2$, 280 µM NADH and cell lysate; the reaction was initiated by the addition of 2-mercaptoethanol to a final concentration of 939 µM. MnSOD activity was determined after incubating the cell lysates at 4°C for at least 1 h with 50 mM sodium cyanide (18). The final

concentration of sodium cyanide in the assay mixture was 5 mM. For catalase and DT-diaphorase assays, cell lysates were prepared as described by Lu et al. (20). Briefly, cells were washed three times with PBS and lysed directly on the plate with a solution containing 0.25 M sucrose, 10 mM Tris-HCl pH 7.5, 1.0 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 1.0% Triton X-100. After incubation at 4°C for 30 min, the lysates were centrifuged at 6,000 x g. The supernatant fractions were collected and stored at -80°C until assayed. Catalase activity was measured as the decrease in absorbance of H_2O_2 at 240 nm according to the method of Lu et al. (20). The assay mixture consisted of 35 mM potassium phosphate, pH 7.2, 0.02% Triton X-100 and 0.03% H_2O_2. The reaction was initiated by addition of cell lysate containing 40-50 µg/ml of protein. Initial reaction velocities were measured. DT-diaphorase activity was measured by the NADPH reduction of 2,6-dichlorophenol-indophenol (DPIP) at 600 nm based on a modification of the method of Ernster (21). Assay mixtures contained 40 mM Tris-HCl, pH 7.8, 250 µM NADPH, 40 µM DPIP and 0.07% BSA, with or without 50 µM dicumarol. The reaction was initiated by the addition of NADPH. Dicumarol specifically inhibits DT-diaphorase and was used to correct for other diaphorase activity in the cell lysates.

Measurements of oxidative damage. Lipid peroxidation was measured as described by Matthews et al. (10). Briefly, cells were washed in PBS and lysed with 1% SDS in the presence of 50 µM butylated hydroxytouluene. Thiobarbituric acid was added to a final concentration of 0.5% and the samples were heated at 95°C for 30 min. After extraction into 5% HCl in butanol, malondialdehyde levels were measured fluorometrically (excitation 528 nm, emission 548 nm). The protocol for assessing oxidative damage to proteins was kindly supplied by G. Perry (Case Western Reserve University) (22). After treatment, the MCF-7 cells were collected by centrifugation and snap-frozen. Cryostat sections (6 µM) were fixed in methacarn (chloroform:methanol:acetic acid, 30:60:10) at 4°C for 10 min., rehydrated through graded alcohol to 95% and incubated with 3% hydrogen peroxide in methanol for 30 min. at room temperature. The sections were passed through graded alcohol starting at 70% and ending in 50 mM Tris-buffered saline, pH 7.6 (TBS). They were then incubated with 0.01% 2,4-dinitrophenylhydrazine in 2N HCl for 1 h at room temperature. After a TBS rinse, non-specific binding sites were blocked with 10% normal goat serum for 30 min. at room temperature. Sections were incubated with rabbit anti-dinitrophenol (DNP) overnight at 4°C, then rinsed briefly in 1% normal goat serum before a 30 min. incubation with goat anti-rabbit IgG. This was followed by a 1 h incubation at room temperature with rabbit-peroxidase anti-peroxidase. Bound antibody was visualized by incubating the sections in 75 mg/ml 3,3-diaminobenzidine/0.015% hydrogen peroxided until a brown color developed. The reaction was stopped by rinsing in TBS. Slides were counter-stained in methyl green.

Sensitivity to oxidative stress. Cells in exponential growth phase were plated in 96-well microtiter dishes (9 x 10^3 cells/well) and allowed to attach overnight. The cells were then exposed for 3 h to hydrogen peroxide or E09. Final concentrations for hydrogen peroxide were 200, 300, 400, 500, 600 or 1200 µM. Final concentrations for E09 were 25, 50, 100, 250 500 and 1000 picomolar. E09 was kindly supplied by Dr. Hans R. Hendricks of the European Organization for Research and Treatment of Cancer, New Drug Development Office. E09 was prepared as a 20 mM stock in DMSO and diluted in culture medium. Control cells were treated with DMSO at the same concentration used for the 1000 picomolar E09 test. Viability was determined three days later using the sulforhodamine B assay.

Viability assays. In one assay, viability was measured using the protein-binding dye, sulforhodamine B (23). At the end of the treatment period, the cells in the microtiter plates were washed two times with PBS. Ice cold TCA (100 µl) was added to each well and the plates were incubated at 4°C for 30 min. The TCA was removed by washing the plates ≈4 times with tap water and excess water was removed by air-drying. Thirty microliters of sulforhodamine B was added to each well and the plates were incubated at room temperature for 20 min. After 3 rinses with 1% acetic acid, the protein-bound sulforhodamine B dye was solubilized in Tris base (50 mM, 100 µl/well) by shaking the plate on a rotary platform for 5 min. Absorbance was read at 540 nm using a microplate reader. In a second assay, viability was determined by direct cell counts. After treatment, cells were collected from the supernate and monolayer and stained with eosin Y. The total number of cells that excluded the dye was determined using a hemacytometer.

Measurements of apoptosis. After treatment, samples to be analyzed by nick end-labeling were collected by centrifugation, washed in PBS and fixed in 4% neutral-buffered formalin for 4 h at 4°C. The fixed cells were collected by centrifugation and embedded in cytoblock gel (Shandon, Inc., Pittsburgh, PA) according to the manufacturer's instructions. The gelled pellet was embedded in paraffin and sectioned onto microscope slides. The sections were baked at 60°C for 1 h and de-paraffinized through graded alcohol. DNA fragmentation was assessed by the terminal deoxyribonucleotidyl-mediated dUTP-biotin nick end labeling assay, using a kinetic-mode instrument (Ventana Medical Systems, Tucson, AZ). For analysis of apoptosis by flow cytometry, cells were incubated in the absence or presence of 1600 U/ml TNF for three days. Floating and attached cells were collected, combined and incubated with 7-amino actinomycin D for no more than 20 min. at room temperature. Samples were analyzed by flow cytometry as described previously (24). To prepare cytospins, cells collected after treatment were deposited onto bovine serum albumen-coated microscope slides, using a Cytospin 2 centrifuge (Shandon, Pittsburgh, PA). The slides were air-dried for 5 min., fixed and stained using a Dade Diff-Quick staining kit (VWR, Phoenix, AZ) according to the manufacturer's instructions. The percent apoptosis was determined based on morphological appearance of cells when examined under a 100x oil immersion lens (1000x magnification). To prepare 1 µM sections cells were collected by centrifugation and fixed in a solution of 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 for 1 h at room temperature. They were then washed three times in 0.1 M sodium phosphate buffer, pH 7.2, embedded in epoxy and cut into 1 µM sections. The sections were stained with toluidine blue. Percent apoptosis was determined as described above.

RESULTS AND DISCUSSION

Expression of antioxidant defense enzymes. Part of the first specific aim of this project was to determine whether antioxidant defenses are altered during TNF-induced apoptosis of breast cancer cells. Northern blot hybridization analysis completed in the first two years of the project demonstrated that mRNA levels for MnSOD increased with TNF treatment of MCF-7 cells, but levels of CuZnSOD, catalase and thioredoxin remain unchanged. We also used biochemical assays to determine whether SOD and catalase activities were altered. There appeared to be no change in catalase activity with TNF

treatment. For SOD activity, there appeared to be a small increase in activity in cells treated with 1000 U/ml TNF for 3 day. The increase was not significant, however. This seemed surprising, given the increase in MnSOD mRNA demonstrated by the northern blot hybridization analyses. This past year we continued to analyze SOD activity with TNF treatment of MCF-7 cells, including a CuZnSOD inhibitor in the assays so that we could determine whether MnSOD was changing. In these experiments, the cells were treated with 1600 U/ml TNF for 2 days before being analyzed. As shown in Fig. 1, total SOD was found to increase by approximately 30% under these conditions. Based on the activity measured when CuZnSOD was inhibited with sodium cyanide, it appeared that the majority of this increase was due specifically to MnSOD. This represents a 5-fold increase in MnSOD activity with TNF-treatment. Treatment of MCF-7 cells with 1600 U/ml TNF for 2 days did not change catalase activity. Activities in the control and treated cells were 8.2 ± 0.2 and 8.7 ± 0.6 μmol/mg/min, respectively.

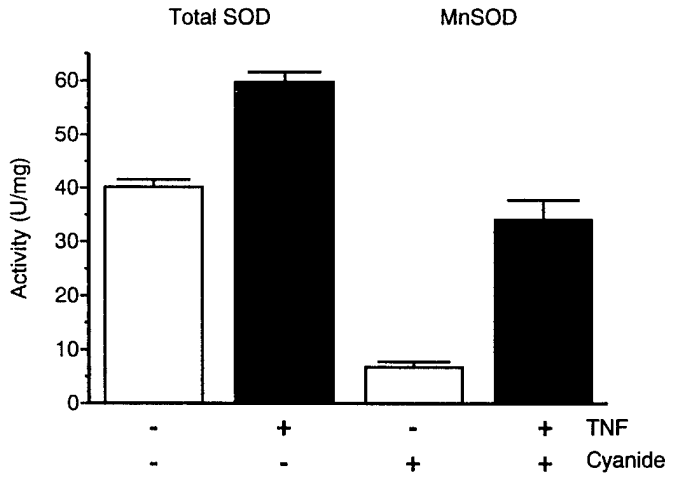

Figure 1. TNF increases MnSOD activity in MCF-7 cells. Lysates prepared from cells grown in the absence or presence of 1600 U/ml TNF for 2 days were tested for total SOD activity, based on the inhibition of oxidation of NADH by superoxide anion. The assays were then repeated with sodium cyanide in the reaction mixture to inhibit CuZnSOD. The experiment was performed in triplicate and error bars indicate SE.

The reason for the discrepancy in total SOD activity results between the earlier and later experiments is not entirely clear. It may be due to a change in the assay protocol. The protocol for measuring MnSOD called for extensive dialysis of cell lysates. Previously, lysates for measuring total SOD were not dialyzed but were passed through a Sephadex G-25 spin column. The molecular weight cut-off for the dialysis tubing we used is 12-14 kd. Perhaps the dialysis step removed a factor in the 14-25 kd size range that previously inhibited total SOD activity in the TNF treated samples.

Evidence of oxidative damage. The remaining part of the first specific aim was to assess the extent of oxidative damage during TNF-induced apoptosis. Oxidative damage to lipids was assessed by measuring malondialdehyde levels. As shown in Fig. 2A, a small increase was seen only after treatment for 2 days with a high dose of TNF (5000 U/ml). The large increase seen in the positive control treated with Fe/ascorbate indicated that the assay was working. Based on this result, the experiment was repeated with enough samples at the 5000 U/ml dose to determine whether the change was significant. In this case, however, average malondialdehyde levels were lower rather than higher in treated vs. untreated cells (Fig. 2B). This suggests either that lipid peroxidation is not occurring as the result of TNF treatment or it is occurring at a level that is not detectable within the range of the assay. The latter possibility is not

unreasonable, since extensive lipid peroxidation would likely cause sufficient membrane damage to result in death by necrosis rather than apoptosis. It may also be difficult to measure lipid peroxidation during apoptosis because of relatively few cells in the population being apoptotic at the same time. This possibility is discussed further below.

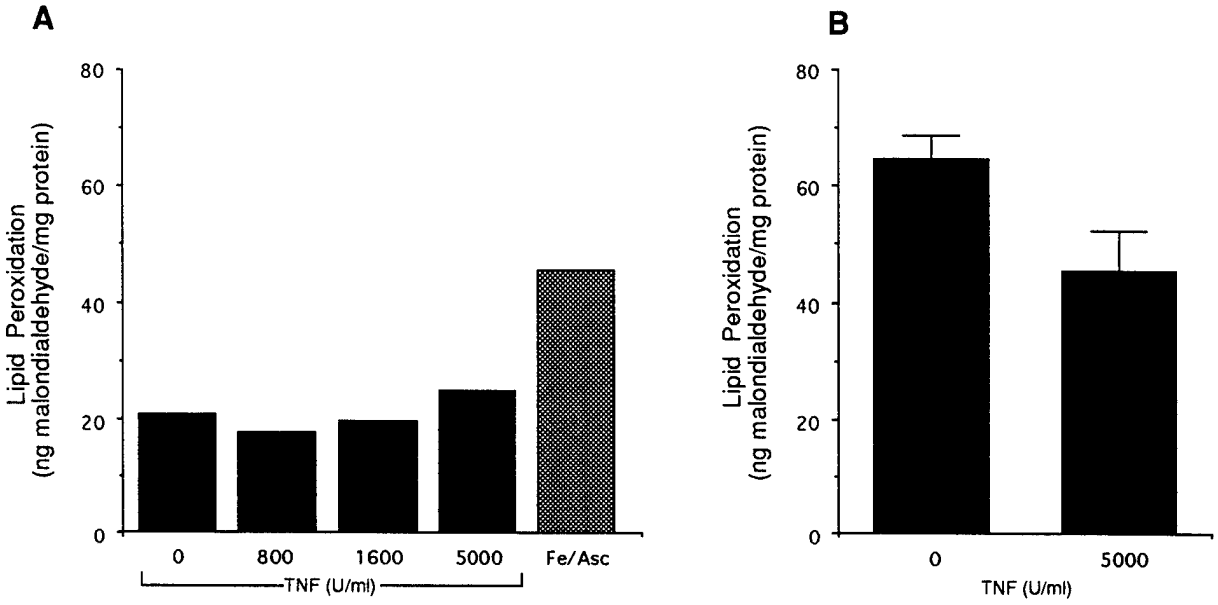

Figure 2. Test for lipid peroxidation in TNF-treated MCF-7 cells. A) Cells were treated with the indicated dose of TNF or with Fe/Ascorbate. The latter treatment was used as a positive control. Malondialdehyde was measured in the cell lysates based on reaction with thiobarbituric acid. B) The experiment was repeated with more samples for the control (n=4) and a 5000 U/m TNF treatment (n=6). Bars indicate average values ± SE.

Oxidative damage to proteins leads to the formation of carbonyl groups (25). To test for oxidative damage to proteins in TNF-treated cells, our plan was to use a method previously described for detection of carbonyl groups by western blotting. Upon reaction with 2,4-dinitrophenylhydrazine (DNP), the carbonyl groups can be detected with an anti-DNP antibody (26). In 1996, Dr. G. Perry and his colleagues reported on experiments using an anti-DNP antibody to detect oxidative damage in brain tissue sections from Alzheimer's patients (22). An advantage of this method is the preservation of cell structure. We obtained a copy of the protocol used by Dr. Perry's group and applied it to control and TNF-treated MCF-7 cells. Initial results with this method in our system are encouraging, it appears as if more antibody has bound to the TNF-treated samples (data not shown). We plan on repeating these experiments after obtaining tissue sections from an Alzheimer's brain tissue to use as a positive control.

Analysis of transfectants. The goal of the second specific aim of this project was to test the importance of oxidative stress to the mechanism of TNF-induced apoptosis through analysis of cells expressing increased levels of antioxidant defense enzymes. Establishing and analyzing stably transfected cells is requiring significantly more time than anticipated. In part, this is because of the different parts of the analysis which must be completed for enough transfectants to be confident of the trend. Analysis includes

confirming that the isolated clones express increased levels of the transfected antioxidant defense gene, testing whether other antioxidant defenses have been altered, determining whether the cells have altered resistance to oxidant stress and then testing for TNF sensitivity.

Sets of DT-diaphorase and catalase transfectants have been isolated and we have worked towards completing the analysis of these, as described further below. While we originally planned to establish stable MnSOD transfectants and test TNF sensitivity, results of such a study were recently reported by Oberley and colleagues (27). These researchers have found that increased expression of MnSOD confers resistance to TNF. This finding supports our hypothesis and has led us to decide that establishing MnSOD transfectants would not make the best use of our time and resources.

ANTIOXIDANT DEFENSE ENZYME ACTIVITIES. Initial measurements of DT-diaphorase and catalase activity in the sets of DT-diaphorase and catalase transfectants, respectively, were reported last year. We have now measured catalase and total SOD activity in the DT-diaphorase clones. SOD activity was found to be lower in the DT12 and DT15 transfectants (Table 1); catalase activity is also lower in DT15. One of these, DT15, has the highest increase in DT-diaphorase activity among the clones. The analyses of control transfectants provides a note of caution. Although these cells were transfected with the empty expression vector (i.e., without DT-Diaphorase cDNA), one of them exhibits higher DT-diaphorase enzyme activity than the parent. Perhaps this reflects some variation within the parent population which becomes apparent with isolation of clones. Having focused on completing the analyses of the DT-diaphorase transfectants, we are just now starting with further analyses of the catalase transfectants.

Table 1. Characterization of MCF-7 clones stably transfected with rat DT-diaphorase

Cells	DT-diaphorase (nmol/mg/min)	Catalase ($\mu M\ H_2O_2$/min/mg)	Total SOD (U/ng)	H_2O_2 IC_{50} (nM)	E09 IC_{50} (pM)
MCF-7	178 ± 1	4.6 ± 1.0	27 ± 2	554	199
neo2	116 ± 7	3.9 ± 0.8	25 ± 6	346	209
neo6	71 ± 6	3.4 ± 0.8	24 ± 7	433	294
neo7	332 ± 60	2.4 ± 0.3	24 ± 6	554	71
DT6	1645	4.4 ± 1.2	25 ± 6	427	39
DT9	4901 ± 77	4.9 ± 1.0	24 ± 4	264	22
DT12	1071 ± 59	5.5 ± 0.6	16 ± 3	563	28
DT15	7400 ± 560	1.9 ± 0.4	17 ± 4	517	19

SENSITIVITY TO OXIDATIVE STRESS. Part of the second Specific Aim of the project is to determine whether the stable transfectants are more resistant to oxidative stresses. We expect that transfection of antioxidant defense genes will make the cells more resistant to the substrate(s) of the protein for which activity has been increased by transfection. The cells may also acquire increased resistance to additional oxidants if other antioxidant defenses are altered, as was seen with total SOD activity in DT12 and DT15. The DT-diaphorase transfectants were tested for sensitivity to two oxidizing agents: E09 and hydrogen peroxide. E09, originally synthesized as an analogue of mitomycin C, has recently entered phase I clinical trials in Europe as an anti-tumor agent. Both mitomycin C and E09 are reductively activated by DT-diaphorase. Sensitivity of a panel of cell lines to E09 has been positively correlated to DT-diaphorase activity (28,29). When we tested the cytotoxic activity of E09 with the DT-diaphorase transfectants, we found that the IC_{50} values indeed correlated with enzyme activity (Table 1). There may be a threshold effect, as there was not much difference in the IC_{50} values between DT9 and DT12, even though DT12 cells have at least 2-fold greater DT-diaphorase activity. There appeared to be little difference in the sensitivity of the different cells to hydrogen peroxide (Table 1). This suggests that the lower catalase activity measured for neo7 and DT15 does not result in increased sensitivity to hydrogen peroxide.

TNF SENSITIVITY. To test our hypothesis that oxidative stress plays a role in the mechanism of TNF-induced apoptosis, reliable assessments of TNF sensitivity are crucial. Thus, we spent a considerable amount of time this past year evaluating approaches for quantitative measurements of apoptosis in our system. Previously, we used an ELISA for histone-associated DNA fragments. This method demonstrated a time- and dose-dependent increase in apoptosis with TNF-treatment, but did not provide information on the percent of the population which was apoptotic. For comparison of apoptosis between different transfectants, we proposed using DNA end-labeling followed by flow cytometry. This method depends upon DNA fragmentation occurring in the apoptotic cells, allowing for end-labeling with fluorescently tagged nucleotides and terminal transferase (30,31). Given the ELISA results, it seemed that this approach should work to label MCF-7 cells in which TNF treatment has led to DNA fragmentation. The advantage of the flow cytometry method over the ELISA is that, ideally, it would provide values for the fractions of the population which are viable, apoptotic or necrotic. There has been a report, however, indicating that terminal transferase-mediated end-labeling method does not always discriminate between apoptotic and necrotic cells (32).

To check whether the end-labeling approach would give reliable results in our system, we treated MCF-7 cells with TNF and processed them for *in situ* analysis (see materials and methods). This approach revealed some apoptotic-appearing cells staining positive for the end-label. However, a significant number of cells with apoptotic morphology that were not positive for the end-labeling reaction were also seen (data not shown). An explanation for this result is that the end-labeling reaction works only in a narrow time window of the apoptotic process induced by TNF treatment of MCF-7 cells. Other cells stained positive for the end-labeling reaction but did not appear apoptotic. We concluded that another method needed to be used for analyzing apoptosis in the transfectants.

Another flow cytometry-based method for measuring apoptosis was reported during the course of our studies (24). The method makes use of 7-amino actinomycin D,

a fluorescent DNA binding agent that discriminates between live, apoptotic and late-apoptotic/dead lymphoid-derived cells treated in various ways to induce apoptosis. Reasoning that the DNA binding agent may operate on a different basis than the end-labeling enzyme, we tested whether it would work in our system. After treatment of MCF-7 cells with various doses of TNF for 3 day, however, we could discern no difference in the populations of control vs. TNF-treated cells by flow cytometry (Fig. 3). We therefore pursued other approaches to measuring apoptosis.

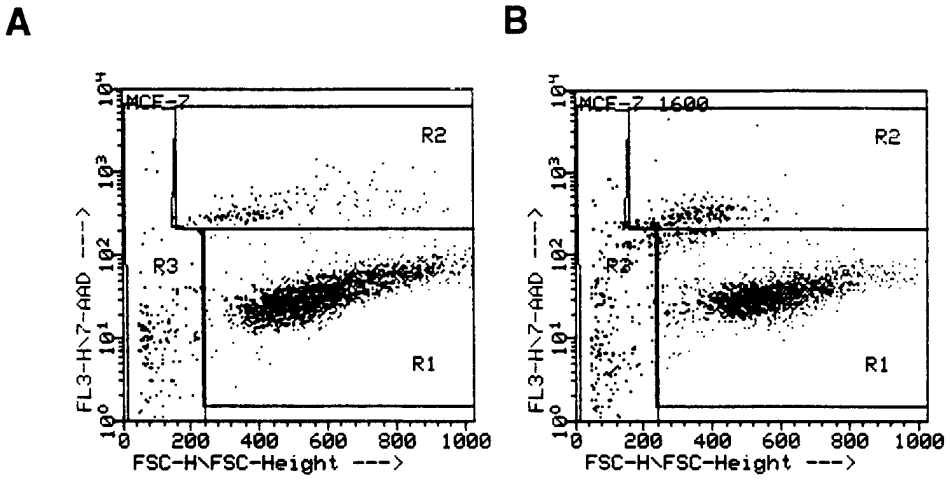

Figure 3. Analysis of MCF-7 cell populations by flow cytometry after staining with the DNA-binding dye 7-amino actinomycin D. A) Control cells; B) Cells treated with 1600 U/ml TNF for 3 days. Fluorescence is plotted on the y-axis and forward light scatter on the x-axis. R1, R2 and R3 indicate live, apoptotic and late apoptotic/dead cells, respectively.

As an alternative to measuring apoptosis by flow-cytometry, we next explored counting apoptotic cells directly. Two different methods were compared. In the first, the cells were used to prepare cytospins and were then fixed and stained with a modified Wright's stain. In the second method, the cells were fixed in 3% glutaraldehyde, embedded in epoxy and cut in 1 μM sections which were stained with toluidine blue. For both methods, the percent apoptosis was determined based on morphological appearance of the cells when examined under a 100x oil immersion lens (1000x magnification), counting at least two slides and 200 cells per slide. When the results of the first method were compared to the ELISA used previously, similar time- and dose-dependent trends were seen (Fig. 4). Comparison of the Wright stain and 1 μM thick sections revealed similar results with these two methods (Fig. 5).

Using the morphological-based assays to measure apoptosis allowed us to determine whether TNF treatment altered mitotic rates in the MCF-7 cells. This is an important factor when considering the effect of TNF on total cell number over time. When we counted the percent mitotic cells in cytospin and 1 μM section slides of MCF-7 cells treated with varying doses of TNF for 24 h, we saw no effect on mitosis (Fig. 6). Thus, we conclude that changes in total cell number with TNF treatment are due to increased death rather than a change in the proliferative rate.

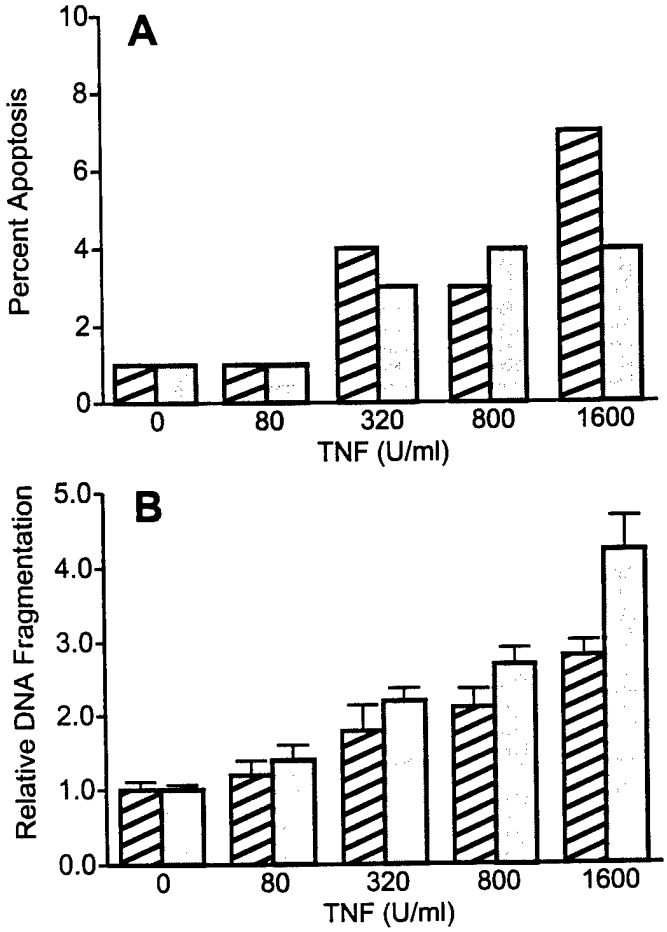

Figure 4. Comparison of different methods for assessing TNF-induced apoptosis of MCF-7 cells. Cells were treated with the indicated dose of TNF for 3 (hatched bars) or 5 (shaded bars) days. A) Apoptotic cells were identified based on nuclear condensation and vacuolization of the cytoplasm in stained cytospin preparations examined with a 100x oil immersion lens. Bars indicate the percent apoptosis averaged from duplicate plates, counting 200 cells in the samples from each plate. B) DNA fragmentation was measured using an ELISA for histone-associated DNA fragments. Relative DNA fragmentation was computed as the ratio of the sample over the control assay reading. Bars indicate the average ratios ± SE from cells tested in triplicate on two separate days.

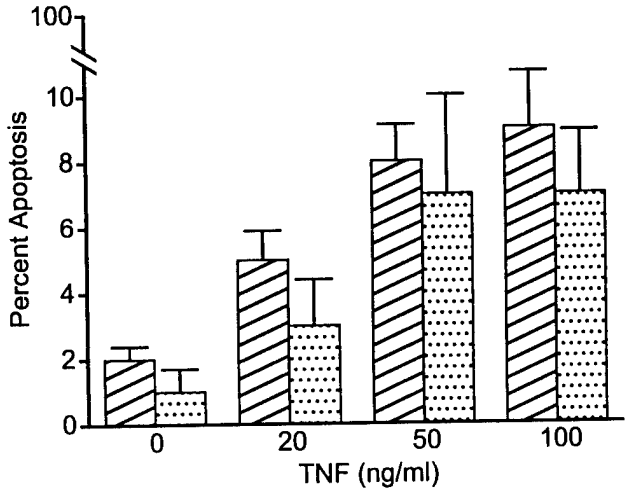

Figure 5. Comparison of different methods for assessing TNF-induced apoptosis of MCF-7 cells. Cells were treated for 24 h with the indicated dose of TNF. (Note: 20 ng/ml TNF is equivalent to 2000 U/ml). One sample was fixed in glutaraldehyde, embedded in epoxy and cut into 1 μM sections which were stained with toluidine blue (hatched bars). A second sample was used to prepare cytospins which were stained with a modified Wright's stain (stippled bars). The percent apoptosis was determined based on morphological appearance of cells when examined under an oil immersion lens. Bars indicate the average value from two slides ± SE.

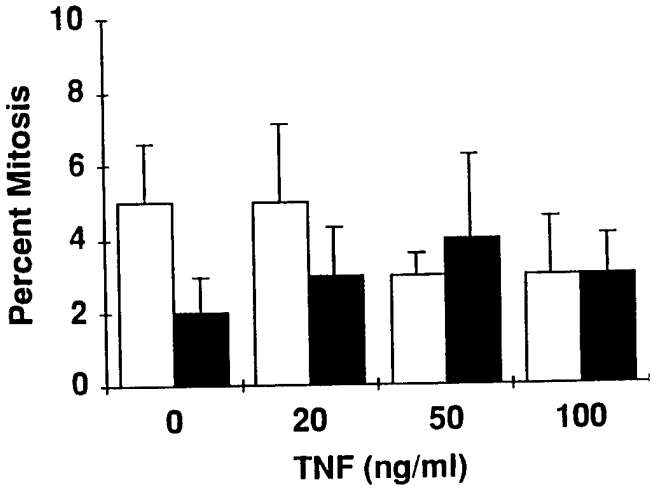

Figure 6. TNF treatment does not alter the mitotic rate. The percent mitosis was determined using the samples described in Fig. 5. Results are from 1 μM sections (open bars) and cytospins (solid bars). Bars indicate the average value from two slides ± SE.

The method of counting apoptotic cells directly was used to examine TNF sensitivity of the DT-diaphorase transfectants. For this experiment, cells were treated with 2000 U/ml TNF for 24 h. The results are given in Table 2, as the percent apoptosis for cytospins. These results suggest that increased expression of DT-diaphorase does not consistently alter TNF sensitivity of MCF-7 cells. However, the increased number of apoptotic cells seen following TNF treatment of DT12 cells is intriguing. Although the increase is small, TNF treatment of this clone also showed greater DNA fragmentation by the ELISA method, compared to the MCF-7 cells and other transfectants, as reported last year.

Since the number of apoptotic cells seen in the samples was small, we next looked at the change in viable cell number over time of TNF treatment. We hoped that this approach would make differences in TNF sensitivity more apparent. For monitoring viable cell number over time we used the sulforhodamine B assay, which is a measure of protein content. Table 2 shows the results of sulforhodamine B assays on MCF-7 cells and DT-diaphorase transfectants treated with 2000 U/ml TNF for 2 days. In general, this method did show greater differences in cell killing than was seen by counting apoptotic cells directly. By this method, DT12 still appears more sensitive to TNF than the MCF-7 parent. There was variation in the replicates of this experiment, especially for the other transfectants. We will repeat it several more time so that we can test for statistical significance.

The sulforhodamine B assay can be used to measure a change in viability over time. It does not, however, directly demonstrate the change in viable cell number. For this, we treated MCF-7 cells with 1600 U/ml TNF and harvested and counted the number of viable cells each day for 5 days. The results are shown in Figure 7 for two different passage numbers. No difference was seen in the slopes of the plots for the different passage numbers. This suggests that sensitivity to TNF is stable for at least 20 passages in culture. As for the kinetics of cell kinetics, the results seemed somewhat surprising; even after 5 days of treatment, many cells remained in the TNF-treated sample. The data also suggest why it may be difficult to document oxidative damage during apoptosis. After 5 days of treatment, there is clearly a difference in the number of viable cells between the untreated and treated samples. In microscopic counts of apoptotic cells (Fig. 4A), the fraction of apoptotic cells seen at 3 and 5 days was less than 10%. This suggests that apoptotic cells are lost from the culture, as might be expected. When trying to assess whether oxidative damage was occurring as the result of TNF treatment,

it is likely that the samples we tested did not accurately represent the apoptotic population.

Table 2. Response of DT-diaphorase transfectants to TNF

	Apoptosis (%) - Cytospins		+ TNF Viability (%) - SRB assay	
Cells	- TNF	+TNF	Replicate 1	Replicate 2
MCF-7	0.5	3.5	81	80
neo7	0.5	1.5	112	78
DT6	2.5	3.0	77	64
DT9	0.5	3.5	59	46
DT12	1.0	6.0	44	50
DT15	1.5	4.0	97	55

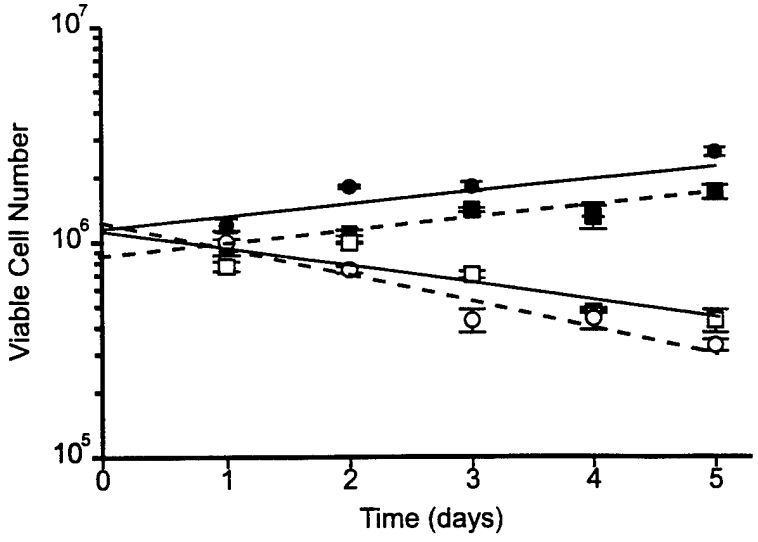

Figure 7. Change in viable cell number of time of TNF treatment. MCF-7 cells at passage number 153 (circles and solid line) or 173 (squares and dotted line) were incubated in the absence (filled symbols) or presence (open symbols) of 1600 U/ml TNF. Attached cells were collected from samples on days 1-5. Viable cell number, based on exclusion of eosin Y dye, was determined with a hemacytometer.

Antisense transfections. The third specific aim of the project involves using antisense methods to test the role of oxidative stress in TNF-induced apoptosis. This aim was to have been completed in the third and fourth year of the project. The experimental approach for this specific aim was to be decided by what was learned about the TNF-sensitivity of the MCF-7 cells stably transfected with antioxidant defenses genes. Given that we demonstrated resistance to apoptosis in transfectants with enhanced expression of an antioxidant defense gene, we would determine whether reduced expression of the gene increased sensitivity to TNF. We hope to make progress on this specific aim during the coming year. It still seems reasonable to finish the analyses of the DT-diaphorase and catalase transfectants before starting antisense constructs of these genes. For the set of DT-diaphorase clones, the initial results suggest increased expression of DT-diaphorase does not generally increase resistance to TNF. Given that expression of catalase is protective, we will use the antisense approach to test whether reduced catalase activity increases TNF-induced apoptosis.

Activation of redox sensitive transcription factors. A series of three papers in the 1 November 1996 issue of *Science* reported on the role of NF-κB in TNF-induced apoptosis (33-35). Previously it had been hypothesized that NF-κB activation leads to the expression of genes which contribute to the mechanism of apoptosis (36). Indeed, this had been our reasoning for looking at NF-κB activation in oxidatively stressed MCF-7 cells. Surprisingly, the *Science* papers demonstrated that loss of NF-κB function increases *sensitivity* to apoptosis. This suggests that the activated transcription factor regulates the expression of genes which *protect* the cells from apoptosis.

MCF-7 cells were not used in the three studies mentioned above, raising the question of whether NF-κB affords protection against TNF in these cells. This question was addressed in a study by Cai et al. (37), reported this last year. Using electrophoretic mobility shift assays after a 90 min. treatment with 50 ng/ml TNF, the investigators found that NF-κB was indeed activated in MCF-7 cells. They subsequently isolated clones stably transfected with a mutated IκBα gene. The mutated IκBα protein acts in a dominant-negative fashion to inhibit NF-κB activation. In the stable transfectants, TNF treatment did not result in NF-κB activation or increased expression of MnSOD. In contrast to the reports mentioned above, there was no difference in the cytotoxic effect of TNF between the control and mutant IκBα transfectants.

Given the above studies, it is clear that additional work is needed to sort out the relationship between oxidative stress, redox-sensitive transcription factors and TNF-induced apoptosis of MCF-7 cells. The goal of the fourth specific aim of the project is to examine the effect of oxidative stress on redox-sensitive transcriptions factors, specifically NF-κB and AP-1. The original plan was to complete this specific aim in months 15-24 of the project. Our delay in getting the transcription factor study started is based on my decision to have as the first priority the completion of the analysis of transfectants. Obviously, it is an important specific aim which should be addressed as soon as possible.

CONCLUSIONS

Based on the results of the project obtained to date, we conclude that in the MCF–7 human breast adenocarcinoma cell line:

1) TNF treatment increases the expression and activity of MnSOD - gene expression and activity were increased approximately 11-fold and 5-fold, respectively, in cells treated for 2 days with 1600 U/ml;

2) The expression of other antioxidant defense enzymes, CuZnSOD, catalase, and thioredoxin, is not altered by TNF treatment; catalase activity does not change;

3) Evidence of oxidative damage to lipids cannot be detected within the limits of sensitivity of an assay for malondialdehyde; this suggest that extensive lipid peroxidation does not occur during TNF-induced apoptosis;

4) Measuring apoptosis in response to TNF using methods based on detection of DNA fragmentation in intact cells is problematic;

5) Consistent results for measuring apoptosis are obtained when cells are visualized microscopically in stained cytospin preparations or 1 μM sections or when apoptosis is measured indirectly as a loss in cell viability;

6) A significant fraction of the population remains viable after treatment with 1600 U/ml TNF for 5 days; apoptotic cells are most likely lost from the culture with time; this is an important consideration when assaying for changes occurring in apoptotic cells;

7) Increased expression of DT-diaphorase does not have a consistent effect on TNF sensitivity; one stable transfectant may have increased sensitivity, but a change in total SOD activity was also seen for this clone;

8) Increased activity of DT-diaphorase in a control transfectant suggests that genetic variation within the original population may factor into the phenotype of isolated clones.

Based on results reported by other investigators during the course of our studies, we conclude that increased expression of MnSOD in MCF-7 cells confers resistance to TNF-induced apoptosis.

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